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NATIONAL DEPARTMENT OF DEFENSE SURVEILLANCE FOR INVASIVE STREPTOCOCCUS PNEUMONIAE: ANTIBIOTIC RESISTANCE, SEROTYPE DISTRIBUTION, AND ARBITRARILY PRIMED POLYMERASE CHAIN REACTION ANALYSES

M. K. Hudspeth
T. C. Smith
C. P. Barrozo
A. W. Hawksworth
M. A. K. Ryan
G. C. Gray
for the Streptococcus pneumonia Surveillance Group

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NAVAL HEALTH RESEARCH CENTER
P O BOX 85122
SAN DIEGO, CA 92186-5122

BUREAU OF MEDICINE AND SURGERY (M2)
2300 E ST. NW
WASHINGTON, DC 20372-5300

National Department of Defense Surveillance for Invasive *Streptococcus pneumoniae:* Antibiotic Resistance, Serotype Distribution, and Arbitrarily Primed Polymerase Chain Reaction Analyses

Marie K. Hudspeth, Tyler C. Smith, Christopher P. Barrozo, Anthony W. Hawksworth, Margaret A. K. Ryan, and Gregory C. Gray, for the *Streptococcus pneumoniae* Surveillance Group[a]

Naval Health Research Center, Department of Defense Center for Deployment Health Research, San Diego, California

To provide surveillance among US military personnel and their beneficiaries, 157 invasive *Streptococcus pneumoniae* clinical isolates were collected systematically from 7 large military hospitals between August 1997 and August 1999. The isolates were studied for antibiotic resistance, and 120 were serotyped and subjected to arbitrarily primed polymerase chain reaction (AP-PCR). Fifty (31.9%) of 157 isolates had intermediate or high-level resistance to penicillin, and 15.9% had multidrug resistance. The most common serotypes were 4, 6B, 9V, 14, 19F, and 23F. Those serotypes associated with penicillin resistance were 6B, 9V, 19A, and 19F. Most invasive disease cases were caused by serotypes included in the currently available 23- and 7-valent pneumococcal vaccines. By use of AP-PCR, 4 DNA groups were correlated with health care site ($P \leq .0001$). These results are valuable in assessing appropriate use of antibiotics and vaccines against *S. pneumoniae* in both military personnel and their families.

Streptococcus pneumoniae frequently causes invasive disease among US civilian populations and military personnel and their families. Invasive *S. pneumoniae* is responsible for ≤500,000 cases of pneumonia, 50,000 cases of bacteremia, and 3000 cases of meningitis in the United States annually [1, 2]. Since the emergence of penicillin-resistant strains in the 1960s, *S. pneumoniae* strains have become more difficult to treat [1, 3]. During recent years in the United States, penicillin resistance among invasive strains of *S. pneumoniae* has risen from being unrecognized to 25%, and the prevalence of multidrug resistance is now estimated at 16% [1, 4–7]. Today, active surveillance for drug-resistant *S. pneumoniae* is crucial in determining appropriate empiric therapy and pneumococcal vaccine use [5, 8–11].

Recently, specific *S. pneumoniae* serotypes have been associated with antibiotic resistance. Resistant strains are found predominantly among serotypes 6B, 9V, 14, 19A, 19F, and 23F [5, 9, 12]. Serotyping has been useful for tracking outbreaks of *S. pneumoniae* and is valuable for epidemiologic studies. For example, serotyping studies have revealed the emergence and spread of serotypes 6B and 23F, which originated in Spain and subsequently spread worldwide to countries such as the United States, South Africa, Korea, Iceland, and the United Kingdom [9, 12–14]. Likewise, arbitrarily primed polymerase chain reaction (AP-PCR) detected identical strains of *S. pneumoniae* in Canada, where serotypes 6B, 14, and 19F were differentiated by using pulsed-field gel electrophoresis (PFGE) and AP-PCR [15].

Data are sparse regarding the temporal changes of *S. pneumoniae* infections among military populations; however, limited data reveal increasing public health problems. For example, clinicians at the Walter Reed Army Medical Center in Washington, DC, noted an increased prevalence of penicillin-resistant strains among sterile site isolates of *S. pneumoniae* from none in 1990 to 36.2% in 1994 [8, 16]. In addition, during the winter of 1989–1990, military trainees in Southern California experienced an epidemic of ≥124 cases of pneumonia thought to be due to *S. pneumoniae.* This epidemic triggered mass prophylaxis with benzathine penicillin G and administration of pneumococcal vaccine to thousands of trainees [8, 17]. Subsequently, there have been other epidemics among US military populations at other sites [8]. In this study, sterile site pneumococcal isolates collected from 7 US military health care centers from August 1997 through August 1999 were characterized by susceptibility testing, capsular typing, and AP-PCR analysis, to provide data for designing better public health interventions.

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[a] *Streptococcus pneumoniae* Surveillance Group members follow the text.

Reprints or correspondence: Dr. Marie K. Hudspeth, Naval Health Research Center, DoD Center for Deployment Health Research, PO Box 85122, San Diego, CA 92186-5122 (hudspeth@nhrc.navy.mil).

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Materials and Methods

Study population. In July 1997, collaborators from 7 of the largest Department of Defense medical health care facilities were asked to preserve and ship all sterile body site *S. pneumoniae* isolates and patient demographic data to the Naval Health Research Center (NHRC; San Diego, CA). Specimens shipped to NHRC had the following information provided: the last 4 digits of the patient's social security number, study identification number for linking of data, specimen date (categorized by season), age category (≤2, 3–17, 18–64, and ≥65 years old), sex, county/state of residence, specimen source, and pneumococcal vaccination status. The number of patients enrolled and the patient population at the sites varies little from year to year. In general, these hospitals see more military family members than active duty military personnel. However, retired military personnel and their families also are seen at these health care facilities. The participating sites were Walter Reed Army Medical Center, National Naval Medical Center (Bethesda, MD), Naval Medical Center (Portsmouth, VA), Naval Hospital (Great Lakes, IL), Wilford Hall Medical Center (San Antonio, TX), Naval Medical Center (San Diego, CA), and Madigan Army Medical Center (Tacoma, WA). Hereafter, the sites are referred to as Washington, Bethesda, Portsmouth, Great Lakes, San Antonio, San Diego, and Tacoma, respectively.

Susceptibility testing. *S. pneumoniae* specimens were preserved in tryptic soy broth with 15% glycerol at −70°C until transport. Isolates received at NHRC were confirmed by standard culture techniques [18, 19]. All strains were taken from stock cultures frozen at −70°C, were subcultured twice onto tryptic soy agar with 5% sheep's blood (TSBA; Hardy Diagnostics), and were incubated at 37°C in 5%–10% CO_2 for 24 h, as suggested by National Committee for Clinical Laboratory Standards (NCCLS) guidelines [18, 19]. Isolates were inoculated, and 6 antimicrobial gradient E-test strips (penicillin, erythromycin, ceftriaxone, trimethoprim-sulfamethoxazole [TMP-SMZ], levofloxacin, and vancomycin; AB Biodisk) were placed onto Mueller-Hinton with 5% sheep's blood (MHSB). MHSB plates were incubated at 37°C in 5%–10% CO_2 for 24 h, as suggested by NCCLS guidelines [18, 19], and were interpreted according to the manufacturer's guidelines (AB Biodisk). Multiple drug resistance was defined as resistance to penicillin and 2 additional classes of non–β-lactam antibiotics. The control strains of *S. pneumoniae* ATCC 49619, *Staphylococcus aureus* ATCC 29213, *Haemophilus influenzae* ATCC 49247, and *Enterococcus faecalis* ATCC 29212 were tested in parallel with each E-test sensitivity test run.

The interpretive values for each antibiotic, in order of intermediate- and high- level resistance, respectively, are as follows: penicillin, 0.012–1.000 and ≥2.0 μg/mL; erythromycin, 1.0 and ≥2.0 μg/mL (values for incubation in 5% CO_2); ceftriaxone, 1.0 and ≥2.0 μg/mL; TMP-SMZ, 1.0–2.0 and ≥4.0 μg/mL; levofloxacin, 4.0 and ≥8.0 μg/mL; and vancomycin (susceptible, ≤1.0 μg/mL; no resistance is known).

Serotyping. A single colony from a pure culture of each isolate was streaked onto 2 TSBA plates and was incubated at 37°C in 5%–7% CO_2 for 18–24 h. Serotyping was performed and interpreted by using a modified version of the latex agglutination typing method described by Facklam et al. [20]. All results from the latex agglutination method were confirmed by using the classic Quellung reaction [21].

AP-PCR. With the use of cultures prepared from serotyping, an initial extraction method was used as described elsewhere [22]. A second extraction was performed using the QIAamp DNA mini kit (Qiagen) by following the manufacturer's procedure for blood and body fluid spin protocol. The positive and negative controls were extracted by the same procedure as that used for the isolates. ATCC 49619 *S. pneumoniae* was used as the positive control, and PCR grade water was used as the negative control. PCR amplification was performed by using the single primer M13 core 5′-GAGGGTGGCGGTTCT-3′ (Midland Certified Reagent), the core sequence of phage M13, as described elsewhere [22, 23]. PCR testing was performed at least twice (2 separate extracts prepared from separate cultures) for reproducibility. A 100-kb molecular weight ladder (Amersham Pharmacia Biotech) was used to compare the strains.

Agarose gels were analyzed with a Gel Doc 2000 analyzer (BioRad Laboratories). All specimen analyses of banding patterns and the dendogram were prepared as recommended by the manufacturer (BioRad). Strains with identical banding patterns were considered to be the same strain [15, 22]. Strains were grouped depending on the major branches of the dendogram tree and were assigned a numeric designation. A 0.8% deviation in band position, when compared with the molecular weight ladder, was allowed during comparisons of DNA patterns. DNA relatedness was calculated on the basis of the dice coefficient, as described in the Quantity One Analysis Software manual (version 4; BioRad).

Statistical analysis. Variables evaluated as potentially associated with antibiotic resistance included patient age group, patient sex, infection site (blood, cerebrospinal fluid, or ear), season of infection (winter, spring, summer, or fall), geographic site, and pneumococcal serotype. Univariate testing was initially performed, and factors associated with antibiotic resistance, with $P \leq .15$, were included in multivariable logistic regression models. Collinearity and multicollinearity diagnostics were completed with all possible significant influential variables before analyses were initiated. Saturated models were reduced by backward elimination. Final models included only variables independently associated with antibiotic resistance, at $P \leq .05$. To evaluate the association between AP-PCR type or group and geographic site, we used Fisher's exact test.

Results

From August 1997 to August 1999, 157 sterile-site *S. pneumoniae* isolates were collected from 7 military hospitals nationwide (table 1). Isolates were from blood culture ($n = 154$), cerebrospinal fluid ($n = 2$), and middle ear culture ($n = 1$). The number of samples received varied by site, and most samples were obtained during the fall season (table 1). Most of the samples were from males ($n = 95$). Only 6 (3.8%) of the 157 patients were identified as having been vaccinated with the 23-valent pneumococcal vaccine. All 6 vaccinated patients were in the ≥65-year-old age group.

Susceptibility testing. All 157 isolates were confirmed by optochin sensitivity and bile solubility to be *S. pneumoniae.* Antibiotic sensitivity testing revealed that 50 (31.8%) had intermediate- or high-level resistance to penicillin (table 2). The percentages of strains with resistance to ceftriaxone, erythro-

Table 1. Demographic characteristics of military health care beneficiaries yielding sterile-site *Streptococcus pneumoniae* isolates at 7 US military hospital sites, August 1997 to August 1999.

Characteristic	No. (%) of patients
Age range, years	
≤2	76 (48.4)
3–17	19 (12.1)
18–64	37 (23.6)
≥65	24 (15.3)
Unknown	1 (0.64)
Sex	
Male	95 (60.5)
Female	62 (39.5)
Specimen source	
Blood	154 (98.1)
Cerebrospinal fluid	2 (1.3)
Inner ear	1 (0.7)
Season collected	
Winter	51 (32.5)
Spring	34 (21.7)
Summer	17 (10.8)
Fall	55 (35.0)
Collection site	
Washington, DC	14 (8.9)
Bethesda, MD	12 (7.6)
Portsmouth, VA	2 (1.3)
Great Lakes, IL	4 (2.6)
San Antonio, TX	38 (24.2)
San Diego, CA	34 (21.7)
Tacoma, WA	53 (33.8)

mycin, and TMP-SMZ were 14.0%, 22.9%, and 32.5%, respectively. All isolates were sensitive to levofloxacin and vancomycin. The prevalence of multidrug resistance was 15.9%. Of the isolates that were penicillin resistant, half (25/50) were multidrug resistant.

Univariate analyses suggested that age, geographic site, and pneumococcal serotype were associated with penicillin resistance. Examination of the frequencies revealed that most of the penicillin resistance came from the strains of serotypes 6B ($n = 8$), 9V ($n = 9$), 14 ($n = 5$), 19A ($n = 4$), 19F ($n = 7$), and 23F ($n = 4$). However, multivariable logistic regression modeling revealed the following variables to be strongly and independently associated with penicillin resistance: specimens from Tacoma (odds ratio [OR], 0.3; 95% confidence interval [CI], 0.1–1.0) and Washington, DC (OR, 4.4; 95% CI, 1.1–18.4) and serotypes 19A (OR 46.5; 95% CI, 4.1–526.1), 19F (OR, 12.5; 95% CI, 3.0–52.1), 6B (OR, 10.2; 95% CI, 2.7–39.3), and 9V (OR 7.5; 95% CI, 2.1–26.7).

When we evaluated factors associated with multidrug resistance, univariate analyses suggested that age, season, and pneumococcal serotype should be included in multivariable modeling. The final logistic regression model revealed that fall season had a significant association (OR, 3.3; 95% CI, 1.2–8.8), and specimens with serotype 19A (OR, 6.7; 95% CI, 1.0–46.6) had borderline statistically significant association with multidrug resistance.

Capsular typing. In all, 18 different serotypes were identified among the 120 isolates studied. Pneumococcal serotypes 4, 6B, 9V, 14, 19F, and 23F were the most common (table 3). Of the 18 serotypes, 15 are included in the currently available 23-valent vaccine, and 7 of these serotypes are in the conjugated pediatric vaccine (table 3). Evaluation of case serotype results by age group revealed that 94% (51/54) of the cases in very young children (≤2 years old) were due to serotypes included in the pediatric 7-valent pneumococcal vaccine. In fact, 82% (98/120) of all typed cases were due to serotypes included in the 7-valent vaccine. Examining serotypes relevant to the 23-valent pneumococcal vaccine revealed that 83% (19/23) of typed cases among adults ≥65 years old and 91% (109/120) of all typed cases were due to serotypes in the 23-valent vaccine.

Molecular typing. The same 120 isolates used for serotyping also were studied with AP-PCR. The dendogram constructed from the AP-PCR analysis revealed 4 groups (table 4). There was 62.2% similarity among all strains (SD, 2.9). The overall range of the number of bands in the dendogram was 4–15 bands (mean, 8.5 bands; SD, 2.3 bands). The strains in PCR groups 1–4 were 89.1% (SD, 2.6%), 90.5% (SD, 4.1%), 84.7% (SD, 3.1%), and 82.9% (SD, 3.6%) similar, respectively. The ranges of the number of bands in the dendogram for groups 1–4 were 5–15 bands (mean, 8.6 bands; SD, 2.0 bands), 5–9 bands (mean, 7.4 bands; SD, 1.3 bands), 4–13 bands (mean, 9.0 bands; SD, 2.3 bands), and 4–15 bands (mean, 8.2 bands; SD, 2.7 bands), respectively. The AP-PCR typing patterns were not statistically associated with penicillin resistance ($P = .28$, Pearson χ^2); however, there was a statistically significant association of AP-PCR group and geographic site ($P \leq .0001$, Fisher's exact test). All 16 isolates from San Diego were in AP-PCR typing group 1; 95.3% of strains (41/43) from Tacoma and 90.3% of strains (28/31) from San Antonio were in AP-PCR typing groups 3 and 4; all 4 isolates from Great Lakes were in group 3; Bethesda had 83.3% of isolates (10/12) in group 2; and both isolates from Portsmouth were in group 1 (table 4).

Discussion

Because of recent pneumococcal pneumonia outbreaks and an apparent increase in the prevalence of antibiotic-resistant

Table 2. Susceptibility of sterile-site *Streptococcus pneumoniae* isolates, by military health care site.

Military site	Total isolates	Penicillin-resistant isolates[a]	Multidrug-resistant isolates[b]
Washington, DC	14	7	1
Bethesda, MD	12	4	3
Portsmouth, VA	2	1	1
Great Lakes, IL	4	0	0
San Antonio, TX	38	13	7
San Diego, CA	34	12	4
Tacoma, WA	53	13	9
Total (%)	157	50 (31.8)	25 (15.9)

[a] Includes intermediate- and high-level resistance to penicillin.

[b] Intermediate- or high-level resistance to ≥2 drug classes plus penicillin, erythromycin, sulfamethoxazole-trimethoprim, or vancomycin.

Table 3. *Streptococcus pneumoniae* serotyping, by military health care site.

Serotype	Washington, DC	Bethesda, MD	Portsmouth, VA	Great Lakes, IL	San Antonio, TX	San Diego, CA	Tacoma, WA	All sites, no. (%)
1[a]							1	1 (0.8)
3[a]					1	1		2 (1.6)
4[a,b]			1	1	6		6	14 (11.7)
6A	1						3	4 (3.3)
6B[a,b]	1	2	1		2	3	5	14 (11.7)
9V[a,b]	3	1		2	6		4	16 (13.3)
10B					1			1 (0.8)
14[a,b]	5	3		1	6	3	9	27 (22.5)
15B[a]		1						1 (0.8)
18C[a,b]						1	4	5 (4.2)
19A[a]					2		3	5 (4.2)
19F[a,b]		2			1	4	5	12 (10.0)
22A		1						1 (0.8)
22F[a]						1		1 (0.8)
23B[a]					1			1 (0.8)
23F[a,b]	2	1			2	3	2	10 (8.3)
33A					1			1 (0.8)
99[c]		1			2		1	4 (3.3)
Total	12	12	2	4	31	16	43	120

NOTE. Data are no. of isolates ($n = 120$).
[a] Serotypes in 23-valent pneumococcal vaccine.
[b] Serotypes in 7-valent conjugated pediatric vaccine.
[c] Untypeable strain.

strains, the NHRC established surveillance for invasive *S. pneumoniae* at 7 US military health care facilities in 1997. The proportion of all invasive cases that the 157 isolates actually captured is not known but is thought to be high. Among isolates, geographic site and serotype were found to be risk factors associated with penicillin resistance. The distribution of serotypes was similar to that of civilian health care facilities, except that, in this study, strains with serotype 14 were not statistically associated with penicillin resistance ($P = .10$, Fisher's exact test) [5, 9]. The prevalence of strains with penicillin-resistant serotypes varied by geographic site, as seen in other studies [4, 5, 9]. Isolates from Washington, San Antonio, Bethesda, and San Diego had a higher percentage of strains with penicillin-resistant serotypes. Serotype 19A and isolates collected in the fall were more associated with multidrug resistance. Serotype 19A was associated previously with penicillin and multidrug resistance [12]. The variation in the prevalence of resistant strains observed at the different military health care sites may be due to overuse of β-lactam antibiotics at some sites. Previous studies have shown that overuse of antibiotics can result in a higher prevalence of antibiotic resistance [4, 14, 24]. Surveillance for antibiotic resistance is crucial for clinicians considering empirical antibiotic therapy [1, 16, 24, 25].

Past studies have shown that serotype and penicillin resistance are associated with DNA profile [9, 26]. In this study, geographic site was the only parameter associated with AP-PCR typing data ($P \leq .0001$, Fisher's exact test). Surveillance and characterization of *S. pneumoniae* strains by genetic analysis, such as AP-PCR and PFGE, have shown that *S. pneumoniae* strains associated with outbreaks are clonal [9, 12–13, 15]. Since the strains in this study did not have identical DNA profiles, our data did not suggest that an outbreak had occurred. A database for PFGE has been developed for the determination of internationally resistant clones; however, there is no similar database for AP-PCR typing. Thus, it was not possible to determine whether any of these strains were international clones. Further surveillance studies are needed to correlate AP-PCR typing with the PFGE national database.

Most isolates (109 [91%] of 120) had *S. pneumoniae* serotypes included in 23-valent pneumococcal vaccine. Of the 23 typed pathogens in adults ≥65 years old, 19 (83%) are in this vaccine. Three patients (serotypes 1, 9V, and 19A) were vaccinated with pneumococcal vaccine before their illness and may be considered to have had vaccine failure. If the other patients ≥65 years old were given the 23-valent vaccine, according to current guidelines [3], and if vaccination was 65% efficacious against invasive disease [27], then ≤10 more cases (8% of all cases) presented here might have been prevented.

Perhaps of more importance, 51 (94%) of 54 of the typed

Table 4. Number of invasive *Streptococcus pneumoniae* isolates, by US military health care site and by arbitrarily primed polymerase chain reaction group.

Site	Group 1	Group 2	Group 3	Group 4	Total
Washington, DC	3	3	6	0	12
Bethesda, MD	1	10	0	1	12
Portsmouth, VA	2	0	0	0	2
Great Lakes, IL	0	0	4	0	4
San Antonio, TX	3	0	19	9	31
San Diego, CA	16	0	0	0	16
Tacoma, WA	2	0	13	28	43

pathogens found in children <2 years old were serotypes included in the pediatric conjugated 7-valent vaccine. None of the children were known to have been vaccinated during the surveillance period. However, if vaccines had been used according to new public health guidelines [28] and if they were 97% efficacious against invasive disease [29], then ≤49 more cases (40% of all cases) might have been prevented. In fact, if the 7-valent vaccine were universally used in infancy and provided long-term protection with 97% efficacy, then ≤95 cases (79% of all cases) identified might have been prevented by this intervention alone. Infants born in 2001 are likely to be the first US cohort with a high percentage vaccinated with the 7-valent pneumococcal vaccine.

Department of Defense policies to vaccinate other groups, including healthy young military recruits, with 23-valent vaccines may soon be developed, as directed by research in this area [30]. Ongoing surveillance, as described here, will help define the effect of such changing vaccine policies on the epidemiology of invasive pneumococcal disease in the population of nearly 2 million US military personnel and their families.

Streptococcus pneumoniae Surveillance Group

In addition to the authors, the following persons are members of the *Streptococcus pneumoniae* Surveillance Group: Annette Hamilton (Walter Reed Army Medical Center, Washington, DC), Marianne Jesse (National Naval Medical Center, Bethesda, MD), Jim Blanks (Naval Medical Center, Portsmouth, VA), Stephanie Thorn (Naval Hospital, Great Lakes, IL), Pat Riley and Royce Brockett (Wilford Hall Medical Center, San Antonio, TX), Tim Driscoll (Naval Medical Center, San Diego, CA), and Mary Myers (Madigan Army Medical Center, Tacoma, WA).

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6. AUTHORS
MK Hudspeth; CP Barrozo, MAK Ryan, GC Gray for the *Streptococcus pneumoniae* Surveillance Group

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Naval Health Research Center
P.O. Box 85122
San Diego, CA 92186-5122

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14. ABSTRACT (maximum 200 words)

To provide surveillance among United States military personnel and their beneficiaries, we systematically collected invasive *S. pneumoniae* clinical isolates from seven healthcare sites between July 1997 and August 1999. Antibiotic testing was performed on 157 isolates, and 120 of these isolates were serotyped and subjected to DNA fingerprinting. Fifty (31.9%) of the 157 isolates had partial or full resistance to penicillin and 17.5% had multidrug resistance. The most common serotypes were 4, 6B, 9V, 14, 19F, and 23F. The serotypes associated with penicillin resistance were 6B, 9V, 14, 19A and 23F. Eighty-eight percent of the cases seen were potentially preventable through the use of currently available pneumococcal vaccines. Using arbitrarily primed PCR, we found four DNA fingerprint groups which had a high correlation with healthcare site (p value ≤ 0.0001). These results are valuable in assessing appropriate use of antibiotics and vaccines against *S. pneumoniae* in military beneficiaries.

14. SUBJECT TERMS

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